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Targeting the Vasopressin Type 2 Receptor for Renal Cell Carcinoma Therapy

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Abstract

Arginine vasopressin (AVP) and its type-2 receptor (V2R) play an essential role in the regulation of salt and water homeostasis by the kidneys. V2R activation also stimulates proliferation of renal cell carcinoma (RCC) cell lines *in vitro*. The current studies investigated V2R expression and activity in human RCC tumors, and its role in RCC tumor growth. Examination of the cancer genome atlas (TCGA) database, and analysis of human RCC tumor tissue microarrays, cDNA arrays and tumor biopsy samples demonstrated V2R expression and activity in clear cell RCC (ccRCC). *In vitro*, V2R antagonists OPC31260 and Tolvaptan, or V2R gene silencing reduced wound closure and cell viability of 786-O and Caki-1 human ccRCC cell lines. Similarly in mouse xenograft models, Tolvaptan and OPC31260 decreased RCC tumor growth by reducing cell proliferation and angiogenesis, while increasing apoptosis. In contrast, the V2R agonist dDAVP significantly increased tumor growth. High intracellular cAMP levels and ERK1/2 activation were observed in human ccRCC tumors. In mouse tumors and Caki-1 cells, V2R agonists reduced

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cAMP and ERK1/2 activation, while dDAVP treatment had the reverse effect. V2R gene silencing in Caki-1 cells also reduced cAMP and ERK1/2 activation. These results provide novel evidence for a pathogenic role of V2R signaling in ccRCC, and suggest that inhibitors of the AVP-V2R pathway, including the FDA approved drug Tolvaptan, could be utilized as novel ccRCC therapeutics.

Keywords

Vasopressin; V2R; renal cell carcinoma; OPC31260; Tolvaptan; Caki-1; 786-O; mouse xenograft; dDAVP; cAMP

Introduction

Renal cell carcinoma is the most common cancer of the kidneys, arising from tubular epithelial cells within the renal cortex. It is the eighth leading cause of cancer related fatalities with 15,000 deaths and 65,000 new cases detected per year in the United States [1]. Despite surgical, immunotherapy and chemotherapy interventions, the 5-year survival rate is only 8% for stage 4 RCC patients. RCCs occur in multiple histologically and genetically heterogeneous subtypes of which, ccRCC represents 80% of the cases of RCC [2]. ccRCC tumor cells are highly invasive, metastatic and resistant to radiation and chemotherapy [2]. Hence, a better understanding of the mechanisms that drive ccRCC pathogenesis could help in the development of new, and more effective therapies for ccRCC.

Arginine vasopressin (AVP) or antidiuretic hormone is produced mainly by the hypothalamus and released into the blood from the pituitary. To elicit its actions in target organs, it binds to its type-1 (V1aR and V1bR) and type-2 (V2R) G-protein coupled receptors (GPCRs). V2R is normally expressed in the renal collecting duct, distal tubule and thick ascending limb segments of renal nephrons, where it plays an essential physiological role in regulating water and electrolyte homeostasis [3]. However, ectopic expression of AVP and its receptors has been reported in breast, pancreatic, colorectal and gastrointestinal cancers, and small cell lung carcinoma [4, 5]. While V1R activation has been observed to increase tumor cell growth [6, 7], V2R agonists are generally known to have angiostatic, anti-proliferative and/or anti-metastatic effects in breast, lung and colorectal cancers [8–10]. However, in contrast to these protective roles of V2R, one study reported that V2R activation increased cell proliferation of ccRCC cell lines of human origin *in vitro* [11]. This finding is notable because ccRCC is thought to originate from proximal tubules [12–15], which normally does not express V2R [16].

While it appears that V2R activation may be pro-proliferative in cultured RCC cell lines, it is not known if V2R is expressed and active in human RCC tumors *in vivo*. It is also not known which RCC subtypes express V2R; if AVP-V2R cell signaling regulates RCC tumor growth; or if V2R is potentially a viable target for therapeutic inhibition in RCC. As such, in the current study, we asked whether AVP-V2R signaling is pathogenic in RCC, and tested the therapeutic potential of targeting V2R for inhibition in this malignancy both *in vitro* and *in vivo*.

Materials and Methods

Drugs and reagents:

Tolvaptan (OPC41061, 7-chloro-5-hydroxy-1-[2-methyl-4-(2-methylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1H-1-benzazepine), OPC31260 (Mozavaptan, 5-(Dimethylamino)-1-[4-(2-methylbenzamido)benzoyl]-2,3,4,5-tetrahydro-1H-benzazepine hydrochloride), 8-Br-cAMP, and dDAVP (1-desamino-8-d-arginine vasopressin) were from Sigma Millipore (St. Louis, MO). Primary antibodies were purchased for phosphorylated cAMP response element binding protein (pCREB) (#9198S), CREB (#9197S), Ki-67 (#9449S), phosphorylated extracellular signal regulated kinase (pERK1/2) (#9101S), and ERK1/2 (#SC-94) from Cell Signaling Technology (Danvers, CA, USA); vascular endothelial growth factor (VEGF) (#SC-7269) and GAPDH (#SC-47724) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and V2R (#V5514) from Sigma Millipore (St. Louis, MO, USA) and CD31 (#ab28364) from Abcam (Cambridge, MA, USA). Secondary antibodies were from Dako (Carpinteria, CA) and ECL reagent was from Perkin Elmer (Waltham, MA, USA).

TCGA data analysis:

TCGA data were accessed and downloaded through the Xena Genome Browser (University of California Santa Cruz). Subsequent analyses were conducted in R (v3.5.1) [17] using the car and Rmisc packages. Figures were constructed using the ggplot2 package.

Human tumor biopsy tissue:

De-identified tissues from surgically removed human ccRCC tumor samples from 8 patients, each of whom had pathologically proven clear cell RCC, and 8 non-malignant control kidney tissues were obtained from the University of Kansas Cancer Center's Biospecimen Core Facility. Our protocols were approved by the Institutional Review Board (IRB).

Tumor Microarray:

TMA array (#KD2082, from US Biomax Inc. Derwood, MD) with 164 ccRCC tumor tissue cores, 8 papillary RCC, 10 other RCC subtypes (6 transitional, 3 chromophobe and 2 squamous cell RCCs), 10 samples each of cancer-adjacent non-malignant control and non-malignant control kidney tissue cores (one specimen per patient). The TMA slide was immunostained for V2R as described below, and V2R staining intensity and immunoreactive areas were scored as 0 (None), 1 (Low), 2 (moderate), or 3 (high). Scoring of tissue cores was performed by a pathologist, blinded to the identity of samples.

Immunostaining:

Tumor tissues were fixed and immunofluorescence and immunohistochemistry staining and imaging were performed as described before [18].

cDNA array:

A human cDNA array (#HKRT102) consisting of cDNA from 9 control non-tumor kidney tissue, 27 ccRCC and 12 papillary RCC was purchased from OriGene, Rockville, MD. RT-

PCR was carried out as following the manufacturer's instructions, and samples were run on 2% agarose gels.

Real-time PCR:

RNA was isolated using a Qiagen RNA isolation kit, cDNA was synthesized using a reverse transcription kit from Applied Biosystems, and PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems, CA). Primers are described in Supplemental Table 1.

Cells:

Human ccRCC cell lines 786-O (ATCC-CLR-1932), Caki-1 (ATCC-HTB46), , ACHN (ATCC-CLR-1611), and human renal proximal tubular HK-2 (ATCC-CLR-2190) cells were from American Type Culture Collection. 786-O cells were authenticated by STR profiling, and Caki-1 cells were tested for mycoplasma contamination.

Cell viability:

Exponentially growing cells in 24-well plates were serum deprived for 16h, following which the cells were incubated with 0.2%FBS- containing media with vehicle or V2R agonist or antagonists for 48h. The cells were then incubated in MTT solution (5 mg/mL) for 2h, following which the MTT solution was washed off and purple formazan solubilized in DMSO and quantified by spectrophotometry at 570nm. The study was replicated 3 times, each with n=6 samples.

Clonogenic assay.

Cells were plated (1,000 cells/well) in 6-well plates. After 16h, cells were treated with different doses of OPC31260. Following 48h incubation, cells were washed and grown for 10 days until visible colonies formed. Colonies were fixed with a 1:1 ratio of acetone: methanol, stained with 0.25% crystal violet in 25% methanol, washed, air-dried and photographed. Crystal violet was dissolved in sodium citrate buffer (0.1M, 50% ethanol, pH 4.2) and absorbance was measured at 590nm. The study was replicated 3 times, each with n=2 samples.

Cell cycle analysis:

Cells grown on 100mm petridishes were serum deprived for 16h to synchronize cells, followed by incubation in 1% FBS-containing media in the presence of vehicle or V2R antagonists for 24h. Cells were then trypsinized, washed twice with PBS, fixed using pre-chilled 70% ethanol overnight, and permeabilized with PBS containing 0.1% Triton X-100, 1 mg/ml propidium iodide (Sigma-Aldrich, MO) and 2 mg/ml DNase-free RNase at room temperature. Flow cytometry was performed using FACS Calibur analyzer (Becton Dickinson, Mountain View, CA), capturing 10,000 events for each sample. ModFit LT TM software (Verity Software House, Topsham, ME) was used to analyze results. The study was replicated 3 times, each with n=2 samples.

Wound closure assay:

Cells were grown to confluence in 6-well plastic plates, then changed to 0.2% FBS-containing medium for 16h. A scratch induced wound was then created using a sterile pipet tip. Plates were washed to remove dislodged cells and the same areas of the wounds were photographed at 0 hour, and after 24h of incubation in 0.2% FBS-containing medium. A separate set of plates with similar treatment was used to assess cell proliferation. The study was replicated 3 times, each with n=3 samples.

Gene silencing using siRNA:

V2R-siRNA (AVPR2-SiRNA) (4390824; Clone 1: S1842, Clone 2: S1843) and Scrambled siRNA (AM4620) were purchased from Ambion. When the cells were 30–50% confluent, transfections were performed using Lipofectamine® RNAiMAX (Invitrogen Carlsbad, CA) following the manufacturer's instructions. For cell cycle analysis, cell viability and wound closure, experiments were started at 32h after transfection and continued as described.

Detection of apoptosis/necrosis using Annexin V-FITC/PI staining:

Annexin V-FITC/PI staining was performed using FITC AnnexinV apoptosis detection kit (BD Biosciences, USA). Similar studies to cell viability were performed. Cells incubated with OPC31260 for 24h were washed in cold PBS, resuspended in calcium containing binding buffer (10 mM HEPES, 140mM NaCl, 5mM CaCl₂; pH 7.4) at 1X10⁶ cells/ml and stained for 15min, with 5µl Annexin V-FITC and 5µl PI at 1 µg/ml. 10,000 cells were analyzed for FITC (excitation 488nm and emission 530nm) and PI (610nm) fluorescence. CellQuestPro® software (Becton, Dickinson, Heidelberg, Germany) was used to determine the percent viable (Annexin V-PI⁻), early apoptotic (Annexin V+PI⁻), late apoptotic/necrotic (Annexin V+PI⁺) and necrotic cells (Annexin V-PI⁺). The study was replicated 2 times, each with n=4 samples.

In vivo xenograft study: All animal studies were carried out according to the protocols approved by the University of Kansas Institutional Animal Care and Use Committee. Female Athymic Nude-Foxn1^{nu} mice, ~25g and 7–8 weeks old from Envigo/Harlan were subcutaneously injected on the right flank region with 1×10⁶ Caki-1 cells in 100µl of DMEM medium. Tumor volumes were measured with calipers following the formula, Tumor volume = (length x width²)/2. When tumor volumes reached ~80–100mm³, mice were randomized based on their tumor volumes using a randomization chart.

The mice received OPC31260 (30mg/Kg BWt), OPC31260 (60mg/Kg BWt), dDAVP (1µg/Kg BWt) by daily intraperitoneal injection[19–21] ; or Tolvaptan (120mg/Kg BWt in 1% aqueous solution of hydroxypropyl methylcellulose) by daily oral gavage for 28 days. Vehicle group received saline (50µl/20g mouse, IP) and 1% aqueous solution of hydroxypropyl methylcellulose (200µl/20g mouse, oral gavage). Body weights and tumor volumes were measured every other day. Investigators were not blinded to the identity of the treatments. At the end of the study, tumors were harvested, photographed, weighed and flash frozen or paraffin embedded for further analysis. 8 mice were used in each group.

Immunoblotting:

Tumors tissues or cultured cells were homogenized in SDS Laemmli buffer and immunoblotting was carried out as described before [22].

Measurement of cAMP:

Tumors were ground to a fine powder under liquid nitrogen and homogenized in 10 volumes of ice cold 0.1M HCl, and centrifuged at 600g. In cell culture studies, Caki-1 cells grown on 6-well plastic plates were pre-treated with IBMX (50 μ M) for 20 minutes, followed by V1aR and V2R antagonists, or dDAVP (1nM [11, 23]) treatment for 10 or 30 minutes. The cells were then washed with ice cold PBS and lyzed in 0.1M HCl. The cAMP levels in tissue and cell extracts were measured as described before [18–20] using an ELISA kit (#CA-200, Sigma-Aldrich, MO, USA). For tumor tissues, 7 control and 8 ccRCC tumor tissue were used. The cell culture study was replicated 3 times, each with n=3 samples.

TUNEL assay for apoptosis:

TUNEL assays were performed on tumor sections using *In Situ* Cell Death Detection Kit (Roche Applied Science, IN, USA) following the manufacturer's instructions. n=8 from each study group.

Statistical analysis:

All statistical analysis was performed using GraphPad Prism, Version 5.0d. Two way repeated measures of analysis of variance (ANOVA) followed by the Bonferroni test, one way ANOVA followed by Dunnett's multiple comparison test, or two-tailed unpaired student's t-test with Welch's correction and F test were performed. $P < 0.05$ was considered significant. Data were expressed as mean \pm SEM for *in vivo* and mean \pm SD for *in vitro* studies. Sample size estimate was not performed for *in vitro* studies. Sample size estimate for *in vivo* studies were made using statistical analysis of power using an on-line calculator at <https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html>. Sample size of 8 mice was determined based on power analysis, to have 95% power, to detect a 50% reduction in tumor weight between Vehicle treated and OPC31260 treated mice ($\alpha = 0.05$). Number of samples and study replicates are provided under each method section.

Results**Abnormal V2R expression in human tumors:**

To determine the possible clinical relevance of V2R expression in cancer, we first examined the pan-cancer gene expression of V2R (AVPR2 gene) and its ligand, AVP, in the TCGA database. AVPR2 was found to be upregulated in cancers of the breast, bladder, colon, lung, ovary, pancreas, prostate, skin, thyroid, thymus, head and neck, and in sarcoma and diffuse large B-cell lymphoma, with kidney cancer being one of the major expressors (Fig-1A). Among the limited number of cancer types covered by the index, higher AVP gene expression was restricted to cancers of the kidney, adrenocortical, bladder and liver (Fig-1B). AVPR2 and AVP gene expression were detected in chromophobe, papillary, and

clear cell RCC (Supplemental-1A, B). Hence, to further examine the role of V2R in RCC, we next determined its expression in human RCC cell lines and tumors.

V2R mRNA expression was detected in 14 out of 27 human ccRCC tumors using a commercially available cDNA array, (Fig-1C), whereas only 1 out of 12 human papillary RCC tumors showed V2R expression (Supplemental-2A). V2R expression was also observed in nearly all cDNA samples that we isolated from primary human ccRCC tumor biopsy samples (Supplemental-2B). Although kidneys are not normally known to secrete AVP, many tumors are known to acquire the capacity to secrete neuropeptides [24]. Hence, to determine if in addition to ectopic V2R expression, ccRCC tumors can also produce its ligand, we examined AVP mRNA levels. AVP expression was detected in 12 out of 27 human ccRCC tumors in the cDNA array (Fig-1C) and 2 out of 8 human ccRCC tumor biopsy samples (Supplemental-2C). AVP was also detected in 5 out of 12 human papillary RCC tumors in the cDNA array (Supplemental-2A). V1R mRNA was detected in all human ccRCC and papillary tumors (Supplemental-2 A,B,E).

Immunostaining to detect V2R protein expression in ccRCC tumor tissue sections showed cell membrane associated V2R in tumor cells, but not in the tumor stroma (Fig-1D). In the non-malignant control kidneys, cells of the proximal tubules did not express V2R (Fig-1D). In a tumor tissue microarray of RCC tumor sample cores from different patients, 156 out of 162 ccRCC samples showed V2R expression (Fig-1E). V2R expression in ccRCC tumor cores also increased with increasing clinical tumor grade (Fig-1F) and TNM values (Fig-1G). V2R mRNA was also detected in Caki-1, 786-0 and ACHN human RCC cell lines (Fig-1H). V1R was found to be expressed in control HK2 cells lines, compared to which, Caki-1, 7860 and ACHN cell lines showed relatively lower V1R expression (Supplemental-2D).

V2R is normally expressed in kidney collecting ducts, distal tubules and thick ascending limbs, but not in proximal tubules [16]. As expected, we detected V2R mRNA in non-malignant control kidney tissue using cDNA array (Fig-1C, Supplemental-2B) and by immuno-staining of tissue sections (Fig-1D, E). V2R was not detected in the HK-2 human proximal tubule cell line (Fig-1H), or in the proximal tubules of non-malignant control kidney tissues (Fig-1D). These results demonstrate that the expression of V2R and its ligand, AVP in human ccRCC tumors is ectopic and abnormal.

V2R antagonists inhibit growth of ccRCC cell lines *in vitro*:

To determine the possible role of V2R in tumor cell survival, proliferation and migration, *in vitro* studies were carried out. Treatment with the V2R antagonist OPC31260 for 48h dose-dependently reduced cell viability of 786-O and Caki-1 cells as determined by MTT assay, with a 50% reduction noted at 5–10 μ M doses (Fig-2A). No significant change in cell viability was noted at 24h under similar treatment conditions. In contrast to ccRCC cells, control HK-2 cells had significantly higher survival (over 92%) at the same doses of OPC31260 (Supplemental-3A). A significant decline in colony formation was also observed starting at 5 and 10 μ M doses of OPC31260 for 786-O and Caki-1 cells respectively (Fig-2B, C). Cell cycle analysis revealed that 60–70% of 786-O and Caki-1 cells treated with 10 μ M or higher doses of OPC31260 were in the G2/M cell cycle phase, suggesting G2/M cell

cycle arrest (Fig-2D, E, F). However, OPC31260 treatment caused only a small increase in apoptosis in these cells (Supplemental-3B, C). OPC31260 treatment also dose-dependently reduced wound repair of 786-O and Caki-1 cell monolayers (Fig-2G, H). These results suggest that inhibition of V2R in ccRCC cells reduces clonogenicity and is cytostatic, and that inhibition of cell proliferation occurs by inducing G2/M cell cycle arrest rather than lytic death.

To further confirm the role of V2R in ccRCC cell growth, we also tested the effect of another V2R antagonist, Tolvaptan. Tolvaptan is a Food and Drug Administration (FDA) approved drug used to treat hyponatremia [25], and was recently approved to treat polycystic kidney disease (PKD) [26]. Caki-1 cells treated with increasing doses of Tolvaptan showed a significant reduction in cell viability (Fig-2I) and wound closure (Fig-2J, Supplemental-3D) starting at a dose of 10 μ M. Treatment of Caki-1 cells with dDAVP, a V2R agonist showed a trend towards increased cell viability and wound closure when compared to vehicle treatment, but the values were not significantly different (Fig-2I, J). Unlike OPC31260, Tolvaptan did not induce G2/M cell cycle arrest. Instead, Tolvaptan treatment at 50 and 100 μ M for 24h showed 18% and 26% increases in the numbers of cells in G0/G1 (Fig-2K).

The V2R antagonist OPC31260 suppressed ccRCC tumor growth in a mouse xenograft model:

To determine if V2R is a therapeutic target for ccRCC, the effect of OPC31260, a V2R antagonist was examined. For the studies, Caki-1 cells, which express V2R (Fig-1C), were implanted subcutaneously in nude mice. Within 10 days, small tumors expressing V2R (Supplemental-4A) appeared. Mice carrying tumors of approximately 80–100mm³ size were randomized into three groups for treatment with vehicle, OPC31260 (30mg/Kg) or OPC31260 (60mg/Kg) for 28 days by daily intraperitoneal injections. Tumor volumes in the vehicle treatment group increased steadily, and by day 28 were 7.8-fold higher than on day 1 (Fig-3A). In contrast, the tumor volumes in the OPC31260 treatment groups failed to even double in size after 28 days of treatment (Fig-3A). At sacrifice, the tumors in the OPC31260 treatment groups were smaller and weighed significantly less than in the vehicle treatment group (Fig-3B, C). No significant difference in tumor volumes or weights were observed between the mice treated with 60 mg/Kg or 30 mg/Kg doses of OPC31260 (Fig 3A, B). These results suggest that V2R could play a pathogenic role in ccRCC, and that its inhibition can reduce tumor growth in mice. OPC31260 treatment did not significantly affect the body weight of the mice, but reduced urine osmolality, as expected, in a dose-dependent manner (Supplemental-4B, C), suggesting that OPC31260 at these doses is well tolerated, but inhibits the V2R-regulated urine concentrating ability in these mice.

Tolvaptan suppressed ccRCC tumor growth, while dDAVP promoted tumor growth in a mouse xenograft model:

To determine if Tolvaptan can be repurposed for RCC therapy, we tested its effect on tumor development in mice. Additionally, the effect of dDAVP, a V2R selective agonist on tumor development was also tested. As before, Caki-1 cells were implanted subcutaneously in nude mice, and following tumor growth, mice were randomized into three groups: vehicle, Tolvaptan (120mg/Kg by daily oral gavage) or dDAVP (1 μ g/Kg by daily IP injection) for 28

days. As in the previous study, the tumor volumes in the vehicle treatment group increased by 8-fold when compared to day 1 (Fig-4A), while in the Tolvaptan treatment group, tumor volumes increased only by 2-fold (Fig-4A). By comparison, tumor volumes in dDAVP treatment group significantly increased starting on day 21, and by day 28 were 14-fold higher than on day 1 (Fig-4A). At sacrifice, the tumors in the Tolvaptan group were smaller and weighed significantly less when compared to the vehicle group, while the tumors were significantly larger in the dDAVP group (Fig-4B, C). Tolvaptan and dDAVP treatment did not significantly change the body weight of the mice, but as with OPC31260 treatment, urine osmolality was significantly reduced by Tolvaptan treatment and increased by dDAVP treatment (Supplemental-5A, B), indicating that these AVP analogs were having their expected effects on urine concentrating ability with minimal obvious toxicity.

V2R antagonists reduced tumor cell proliferation and VEGF levels, and increased apoptosis *in vivo*:

To determine the effect of Tolvaptan and OPC31260 on tumor cell growth, we measured cell proliferation and apoptosis in the tumors obtained from the above *in vivo* studies. In both the OPC31260 and Tolvaptan groups, the tumors showed significantly reduced Ki-67 staining (Fig-5A,B), and increased TUNEL staining (Fig-5A,C) compared to vehicle treatment. In contrast, tumors in the dDAVP group showed a significant increase in Ki-67 staining and reduced TUNEL staining compared to the vehicle group (Fig-5A, B, C). Tumor microvessel density, determined by staining for CD31, was significantly reduced by OPC31260 treatment and increased by dDAVP treatment (Fig-5A, D). Tolvaptan treatment did not appear to alter tumor microvessel density. However, OPC31260 and Tolvaptan significantly reduced vascular endothelial growth factor (VEGF) protein levels, an important angiogenic growth factor in the tumors compared to vehicle treatment (Fig-5E, F), while dDAVP did not affect VEGF levels. These results suggest that V2R inhibition can reduce angiogenesis and tumor cell proliferation, and increase apoptosis in ccRCC tumors. To further test the effect of V2R inhibition on VEGF expression in ccRCC cells, we treated Caki-1 cells with OPC31260 and Tolvaptan. VEGF protein levels were unaffected in Caki-1 cells treated for 16h with dDAVP, but were significantly reduced by Tolvaptan and OPC31260 (Fig-5G).

V2R activity stimulates mitogenic cell signaling and cell growth in ccRCC:

To examine V2R post-receptor mechanisms in ccRCC, we examined human ccRCC tumor biopsy tissue samples. In ccRCC tissue, high levels of pERK1/2 (Thr202/Tyr204), ERK1/2, pCREB (Ser133) and CREB were observed (Fig-6A and Supplemental-6A,B), as well as high intracellular cAMP levels (Fig-6B). Since ERK1/2 can be regulated by multiple mechanisms in cancer cells, including by V1R [7], we determined the effect of V2R antagonists on ERK1/2 activity. In mouse xenograft tumor tissue lysates, a significant reduction in pERK1/2 was observed in the OPC31260 and Tolvaptan groups, compared to the vehicle treatment groups (Fig-6C,D,E,F). We next measured intracellular cAMP generation *in vitro*, since (unlike V1R) activation of V2R induces Gs-mediated adenylate cyclase stimulation resulting in increased intracellular cAMP levels and CREB activity. Short term treatment with dDAVP significantly increased intracellular cAMP levels in Caki-1 cells, while the V2R antagonists OPC31260 and Tolvaptan significantly reduced cAMP levels (Fig-6G). Consistently, dDAVP increased pERK1/2 and pCREB levels in

Caki-1 cells within 5–30 minutes after treatment (Fig-6H), while OPC31260 and Tolvaptan reduced the pERK1/2 and pCREB levels (Fig-6I). In this study, the V1R antagonist SR49059 showed no effect on pERK1/2 and pCREB levels (Fig-6I). Cyclic AMP can either be pro- or anti- proliferative, depending on the type of cancer. To further confirm that an increase in intracellular cAMP induces proliferation in ccRCC cells, we determined the effect of cAMP on cell proliferation using MTT assay. Caki-1 cells were treated with forskolin, a direct activator of adenylate cyclase, that increases intracellular cAMP, or 8-Br-cAMP, a cell permeable cAMP analog. Both treatments significantly increased cell proliferation at 48h compared to vehicle treated cells (Supplemental-7).

To further confirm a role for V2R in ccRCC cell growth, we tested the effect of V2R gene silencing in Caki-1 cells. When compared scrambled-siRNA group, V2R-siRNA or did not show cell cycle arrest (Fig-6J, K), but showed significantly reduced cell viability (Fig-6L) and wound closure (Fig-6M). Importantly, OPC31260 treatment caused G2/M cell cycle arrest, reduced cell viability and wound closure in the scrambled-siRNA group, it failed to do so in the V2R-siRNA group (Fig-6K, L, M) consistent with the effect of OPC31260 being through inhibition of V2R.

Discussion

The present study demonstrates a novel role of the vasopressin type 2 receptor (V2R) in ccRCC tumor growth. Herein, we show for the first time that V2R is abnormally expressed in human ccRCC tumors and plays a pathologic role in driving tumor growth. V2R antagonists were found to reduce cell viability, clonogenicity, and wound closure, and to induce cell cycle arrest of ccRCC cell lines *in vitro*. Moreover, V2R antagonists suppressed ccRCC tumor growth in a mouse RCC xenograft model by reducing tumor cell proliferation and increasing apoptosis, while a V2R selective agonist had the reverse effect. V2R inhibition was found to reduce cAMP levels, and ERK and CREB activity in ccRCC cell lines or mouse xenograft tumors. These studies reveal a previously undescribed role of V2R in the pathogenesis of ccRCC, and suggest the possibility of using V2R antagonists, including the FDA-approved drug Tolvaptan as a novel treatment strategy for ccRCC.

While V2R mRNA levels in the TCGA database did not correlate with ccRCC patient survival, our studies demonstrated that V2R is upregulated and can be implicated in the pathology of ccRCC. A recent report citing TCGA data demonstrated a total of 9 significantly mutated genes in ccRCC. Mutations in the tumor suppressor PTEN correlates with poor survival in the majority of cancer patients. However, mutant PTEN in ccRCC samples in the TCGA database do not correlate with poor survival. Thus, although by far, mRNA levels are indicative of the role of a protein in overall survival of cancer patients, there are instances when they do not correlate despite a role in disease pathogenesis [27]. Our observation of abnormal V2R expression in human ccRCC tumors is novel, and consistent with a previous *in vitro* study which showed V2R expression in cultured ccRCC tumor cell lines [11]. We demonstrated V2R mRNA and protein expression in human primary ccRCC tumors and detected V2R expression in tumor cell membranes. It is to be noted that V2R is normally expressed in the collecting duct, distal tubule and thick ascending limb segments, but not in the proximal tubules of the renal nephrons [16]. Hence

V2R expression in ccRCC tumor cells, which are believed to be of proximal tubular origin [12–15], is ectopic, rather than simply being higher in expression when compared to non-malignant control tissue.

We also detected AVP mRNA expression in human ccRCC tumors. Normally, AVP is synthesized and secreted by specialized neurosecretory cells of the hypothalamus, which have not been described before in normal kidneys, or in ccRCC tumors. Thus, ectopic expression of V2R as well as its ligand could provide a survival and growth advantage to ccRCC tumor cells. ‘Neuropeptide hormones and their receptors are known to be involved in the oncogenic transformation process in various cancers [24]. Ectopic expression of AVP and its receptors has been reported in prostate, colon, breast, small cell lung carcinoma, gastrointestinal, and head and neck cancers [28, 29]. An abnormal splice variant of V2R, which could negatively regulate normal V2 receptors has also been reported [29, 30]. Based on prior studies, the effect of AVP on tumor growth appears to be dependent on the following: (A) Type of cancer: AVP promotes growth of SCLC and breast cancer cell lines *in vitro* [7, 31–33]. *In vivo*, transgenic AVP overexpression increased mammary tumor growth [34], while antibody-mediated targeting of pro- vasopressin impaired SCLC and breast cancer tumor growth in mice [35, 36]. However, in other studies AVP had no growth stimulatory effects on SCLC or adreno-cortical tumor cell lines [37–39]. (B) AVP dose: AVP doses of 0.01–1nM were growth stimulatory, while doses above 10nM were growth inhibitory in MCF7 breast cancer cell lines [33]. (C) AVP receptor subtype and their post-receptor signaling: The growth-promoting role of AVP on cancer cells is thought to be regulated by V1R via G proteins of the Gq family, and attributed mainly to rise in $[Ca^{++}]_i$ and pERK1/2 levels. Moreover, V1R antagonist treatment reduced AVP induced proliferation of breast cancer cells [4], while V1R expression in CHO cells which normally do not express AVP receptors increased their cell proliferation [6] [40]. Unlike V1R, V2R activation is generally thought to inhibit cancer cell proliferation because V2R activation increases intracellular cAMP levels, a known inhibitor of cell proliferation in some cancers [41–45]. However, cAMP is also pro-proliferative in many types of cancers, and in PKD through stimulation of the ERK pathway [46–52].

Our *in vivo* studies showed that dDAVP can stimulate increases in tumor cell proliferation and tumor growth, and a reduction in tumor cell apoptosis. This observation is consistent with an earlier *in vitro* study in which dDAVP increased proliferation of ccRCC cell lines, which could be blocked by a V2R antagonist [11]. However, dDAVP and its modified analogues reduce tumor metastasis, angiogenesis and cell proliferation in lung, prostate, breast and colorectal cancers [8–10, 53–56]. Since dDAVP has greater selectivity for V2R compared to V1R [57], these studies concluded that the effect of dDAVP could be via V2R, and protective in these cancers. Our *in vitro* and *in vivo* studies in ccRCC now show that V2R is growth promoting in ccRCC. While it is currently unclear as to why dDAVP’s role in tumor progression is different in breast cancer or small cell lung carcinoma *versus* ccRCC, the post-receptor cell signaling mechanisms could partly explain the difference.

Our studies show that intracellular cAMP and pERK1/2 levels are high in human ccRCC tumors, and can be increased by dDAVP and suppressed by V2R antagonists *in vitro* and *in vivo*. Since V1R activation does not increase cAMP, and because dDAVP, OPC31260 and

Tolvaptan are selective for V2R, cAMP mediated activation of ERK1/2 is likely to be the key V2R post-receptor mechanism in ccRCC. The RAS-RAF-MEK-ERK signaling cascade can be activated by multiple mechanisms in cancer cells [58, 59] and its inhibition can reduce tumor growth and angiogenesis in rodent models of RCC [60, 61]. Moreover, sorafenib, a multi-kinase inhibitor which inhibits RAF-1 and B-RAF, is an approved drug for RCC [62]. We found V1R to be expressed in all human control kidneys and ccRCC tumors. However, SR49059, a V1R inhibitor, failed to reduce pERK1/2 levels in Caki-1 cells, unlike V2R antagonists which did so. Importantly, V2R gene silencing in Caki-1 cells not only reduced cell viability and wound closure, but abolished the ability of OPC31260 to induce cell cycle arrest or further reduce cell viability and wound closure. These findings suggest that V2R activation can regulate ccRCC tumor growth.

V2R activation in response to AVP is a frequent and normal occurrence in some renal tubular segments, but it does not stimulate cell proliferation or angiogenesis. In fact, dDAVP suppresses cell proliferation related gene expression in normal mouse kidneys [63], because cAMP via PKA inhibits RAF-1, and hence the RAS-RAF-MEK-ERK signaling pathway, which inhibits cell proliferation [64]. The pro-proliferative and tumor growth promoting effects of V2R stimulation in ccRCC in our study is novel, and similar to that in PKD, a disease characterized by abnormal proliferation of renal tubular epithelial cells causing progressive growth of renal cysts. Unlike ccRCC tumors, cysts in PKD originate mainly from collecting ducts [18] which normally express V2R. In PKD, V2R expression, cAMP and pERK1/2 mediated cell signaling are enhanced in a setting of low intracellular calcium levels, which allows PKA activation of B-RAF, stimulating increased cell proliferation [64]. Similarities exist in pathogenic mechanisms in PKD and cancers in general [65], and our study adds another by demonstrating that a V2R, cAMP and ERK1/2 dependent mechanism could be common to PKD and ccRCC.

OPC31260 is about 100 times more selectivity for V2R than for V1aR [66], while Tolvaptan has 29-fold greater selectivity for human V2R than V1aR [67]. Moreover, Tolvaptan, a structural derivative of OPC31260 does not inhibit V1bR and has no intrinsic V2R agonistic effect [67]. In the current study OPC31260 (30mg/Kg) had a relatively better effect on reducing tumor size, tumor cell proliferation and microvessel density and increasing apoptosis compared to Tolvaptan *in vivo*. OPC31260 was also more effective in causing cell cycle arrest, reducing cell viability and wound closure than Tolvaptan. Although Tolvaptan has relatively better oral activity than OPC31260 [67], it has a mean absolute bioavailability of 56% in humans [68]. This could be the reason for the relatively lower effect of Tolvaptan (oral) compared to OPC31260 (intraperitoneal) in our *in vivo* study. OPC31260 and Tolvaptan doses (30mg/Kg, and 120mg/Kg respectively) which significantly suppressed ccRCC tumor growth in our study are known to block AVP-dependent V2R activity and reduce urine osmolality in rodents [66, 69]. A dose of 60mg/Kg of OPC31260 did not show a significantly higher reduction of tumor size, compared to the 30mg/Kg dose possibly due to target saturation at the lower dose. While the Tolvaptan dose of 120mg/Kg was higher than the doses of 60mg/Kg used in normal humans or to treat PKD [68, 70], it should be considered that Tolvaptan treatment is designed to slow kidney enlargement over years or decades of treatment time for PKD, a strategy that is quite different than relatively short term treatments to stop tumor growth. Tolvaptan treatment

could cause side effects including polyuria, thirst or in rare cases, liver toxicity in patients [68, 70]. Although OPC31260 was found to have an IC₅₀ value of 1.2 μM for V2R *in vitro* in isolated rat kidney plasma membranes [66], in our studies, a dose range of 5–10 μM was required to significantly inhibit migration and colony formation and cell viability, and induce cell cycle arrest of Caki-1 and 786-O cells *in vitro*. This could be because higher doses are required in RCC cells to achieve antitumor efficacy. This observation is typical of other repurposed drugs for oncological applications [71, 72].

In our *in vitro* studies, both OPC31260 and Tolvaptan reduced cell viability and wound closure in unstimulated ccRCC cells and reduced tumor growth *in vivo*. In addition, OPC31260 and Tolvaptan also significantly increased tumor cell death by apoptosis, while dDVAP treatment reduced apoptosis compared to vehicle treatment in mouse xenograft tumors. Although the mechanism by which V2R activity regulates tumor cell survival is currently unclear, apoptotic cell death in the V2R antagonist treatment groups could also have contributed to the smaller tumor size. Tolvaptan was recently approved by the US FDA, based on a recent clinical trial for PKD therapy [73]. Thus, while ectopic expression of V2R by ccRCC tumor cells could be an adaptation that gives them a survival and pathogenic advantage, it also makes V2R a potentially useful target of inhibition for ccRCC therapy.

V2R is a homeostatic hormone receptor required for sodium and water balance in the body. Although our study is the first to show a pathologic role for V2R in ccRCC tumor growth, hyponatremia, caused by increased V2R activity, and the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) due to ectopic release of vasopressin are known to occur in hepatocellular, gastric, head and neck, breast and small-cell lung cancer patients [74, 75]. Moreover, hyponatremia is associated with poorer RCC outcomes and has been proposed to be used as a prognostic indicator or predictive factor [76, 77]. Importantly, Tolvaptan is routinely used for the treatment of hyponatremia in these patients [78]. This is consistent with our data, which suggest that the increased AVP in these cancer patients may be the cause for hyponatremia, and could also be promoting tumor growth.

In summary, our studies demonstrate ectopic expression of V2R and AVP in ccRCC. The V2R agonist dDAVP increased cell proliferation, angiogenesis and tumor growth, whereas, V2R antagonists OPC31260 and Tolvaptan suppress tumor growth by reducing tumor cell proliferation, increasing apoptosis and reducing angiogenesis. Although OPC31260 was found to be more effective compared to Tolvaptan, the later is an FDA approved drug for the treatment of hyponatremia and PKD, and could be repurposed for use in ccRCC therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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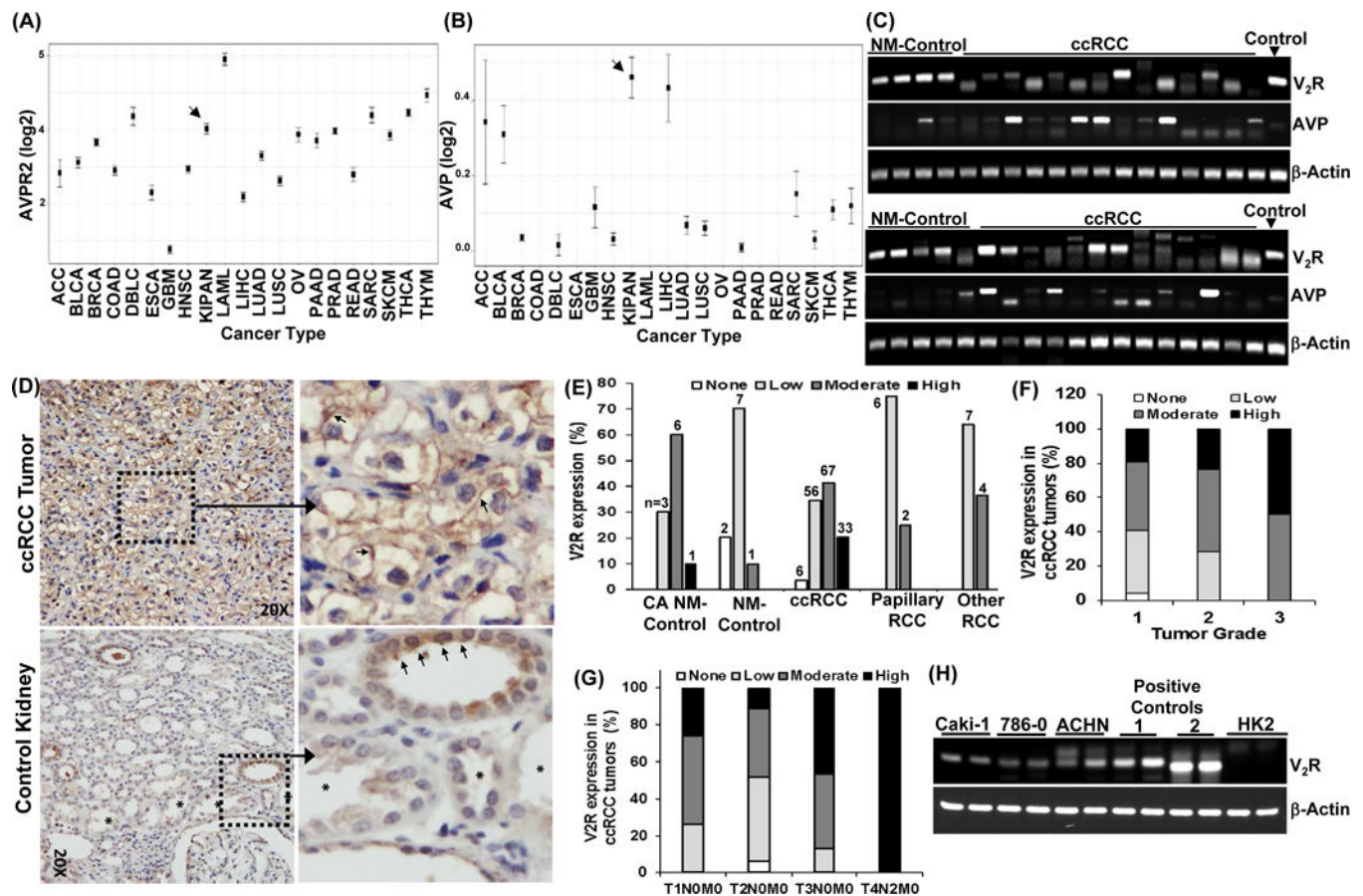


Figure 1. V2R expression and cell signaling in human RCC tumors:

(A) Pan-cancer V2R (AVPR2 gene) and (B) AVP gene expression determined from the TCGA database. Black arrow depicts levels in Pan-kidney cancer (KIPAN). Other tumor types assessed include Adreno Cortical carcinoma (ACC), Bladder (BLCA), Breast (BRCA), Colon (COAD), Diffuse Large B Cell Lymphoma (DLBC), Esophageal Carcinoma (ESCA), Head and Neck (HNSC), Acute Myeloid Leukemia (LAML), Liver Hepatocellular Carcinoma (LIHC), Lung Adenocarcinoma (LUAD), Lung Squamous Cell Carcinoma (LUSC), Ovarian (OV), Pancreatic Adenocarcinoma (PAAD), Prostate Adenocarcinoma (PRAD), Rectum Adenocarcinoma (READ), Sarcoma (SARC), Skin Cutaneous Melanoma (SKCM), Thyroid Carcinoma (THCA), Thymoma (THYM). (C) RT-PCR for V2R, AVP and β -actin using a cDNA array of human non-malignant control (NM-control) kidney and ccRCC tumors. Caki-1 cell and mouse pituitary lysate are used as positive controls respectively for V2R and AVP respectively. (D) Immunostaining for V2R in human ccRCC tumor or NM-control kidney samples. * represents proximal tubules and arrows point to cells showing V2R staining (brown). (E) Pathologist's scoring of V2R expression in tissue microarray. Numbers in the graph represent the number of tissue core samples. (F) The percentage of ccRCC tumors with V2R expression in different tumor grades and (G) different TNM staging. (H) RTPCR for V2R and β -actin in cultured Caki-1, 786-O and ACHN human RCC cell lines. Positive controls 1 and 2 represent cystic epithelial cells from human polycystic kidney disease kidneys and 18 h water-deprived mouse kidneys, respectively. The HK-2 human proximal tubule cell line is a negative control.

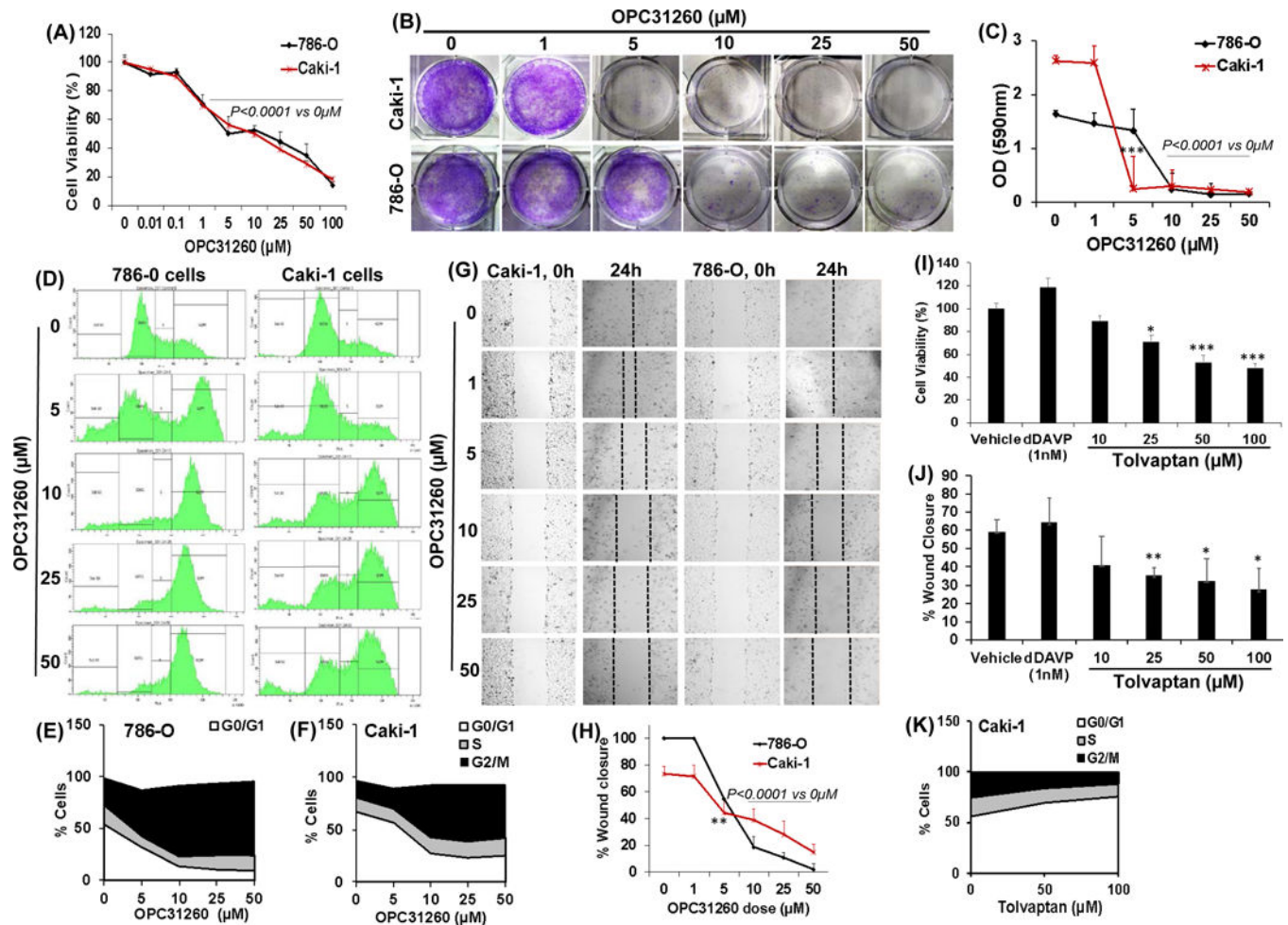


Figure 2. Effect of V2R antagonists on growth of RCC cell lines:

(A) Cell viability of 786-O, and Caki-1 cells treated with OPC31260 for 48h measured by MTT assay. (B) Representative images of clonogenic assays, of Caki-1 and 786-O cell lines treated with OPC31260. (C) Quantification of OPC31260-resistant colony formation. *** $P < 0.001$ vs $0\mu\text{M}$ OPC31260. (D) Representative histograms and (E) quantification of cell cycle stages in 786-O and (F) Caki-1 cells treated with OPC31260 (24h), measured by FACS analysis of propidium iodide stained cells. (G) Representative images of wound healing assay at 0 and 24h after the scratch was placed on monolayers of Caki-1 and 786-O cells in the presence of different doses of OPC31260, and (H) quantitation of their percent wound closure. ** $P < 0.01$ vs $0\mu\text{M}$ OPC31260. (I) Cell viability of Caki-1 cells treated with dDAVP or Tolvaptan for 48h measured by MTT assay. (J) Percent wound closure of Caki-1 cells, 24h after the scratch was placed in the presence of dDAVP or Tolvaptan. (K) Quantification of cell cycle stages in Caki-1 cells treated with Tolvaptan. (L) Representative images for figure K. All error bars represent SD. * $P < 0.05$ ** $P < 0.01$ and *** $P < 0.001$ vs vehicle or $0\mu\text{M}$ V2R antagonist. P values by one way ANOVA followed by Dunnett's multiple comparison test for Fig 2A, 2C, and 2H, and two-tailed unpaired student's t-test with Welch's correction and F test in Fig 2I and 2J.

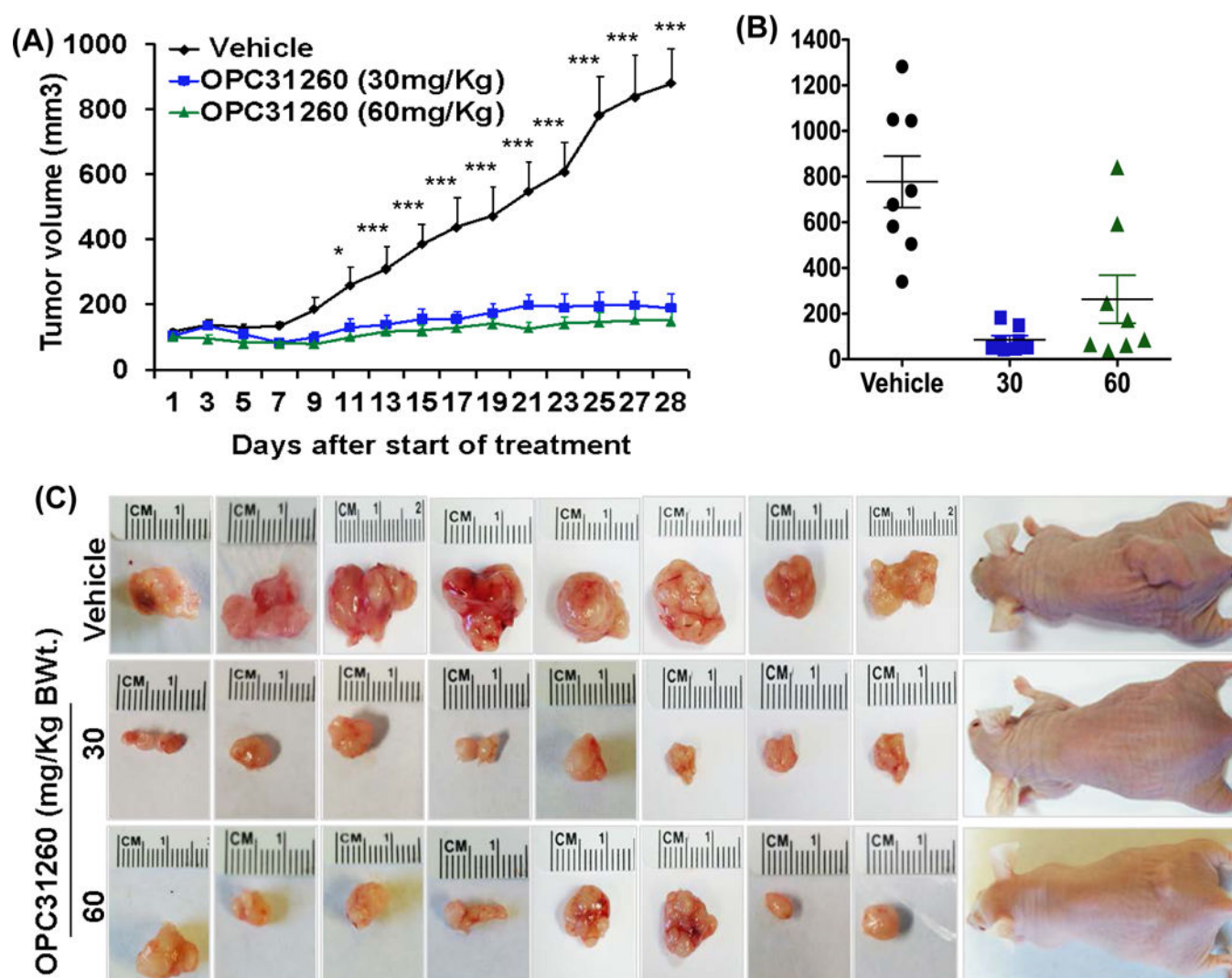


Figure 3. The effect of OPC31260 on RCC tumor progression:

(A) Nu/Nu mice were inoculated subcutaneously with Caki-1 cells, and 10 days later were treated with vehicle or OPC31260 (30 or 60 mg/Kg) for 28 days. Tumor volume is shown.

(B) Images of tumors and mice with tumors at sacrifice. (C) Tumor weights at sacrifice.

* $P < 0.05$, *** $P < 0.001$ vs OPC31260 (30 or 60mg/Kg) in Fig 3A and vs vehicle in Fig 3C, (n=8 mice/group). All error bars represent SEM. P values by two way repeated measures of ANOVA followed by the Bonferroni test for Fig 3A, and one way ANOVA followed by Dunnett's multiple comparison test for Fig 3B.

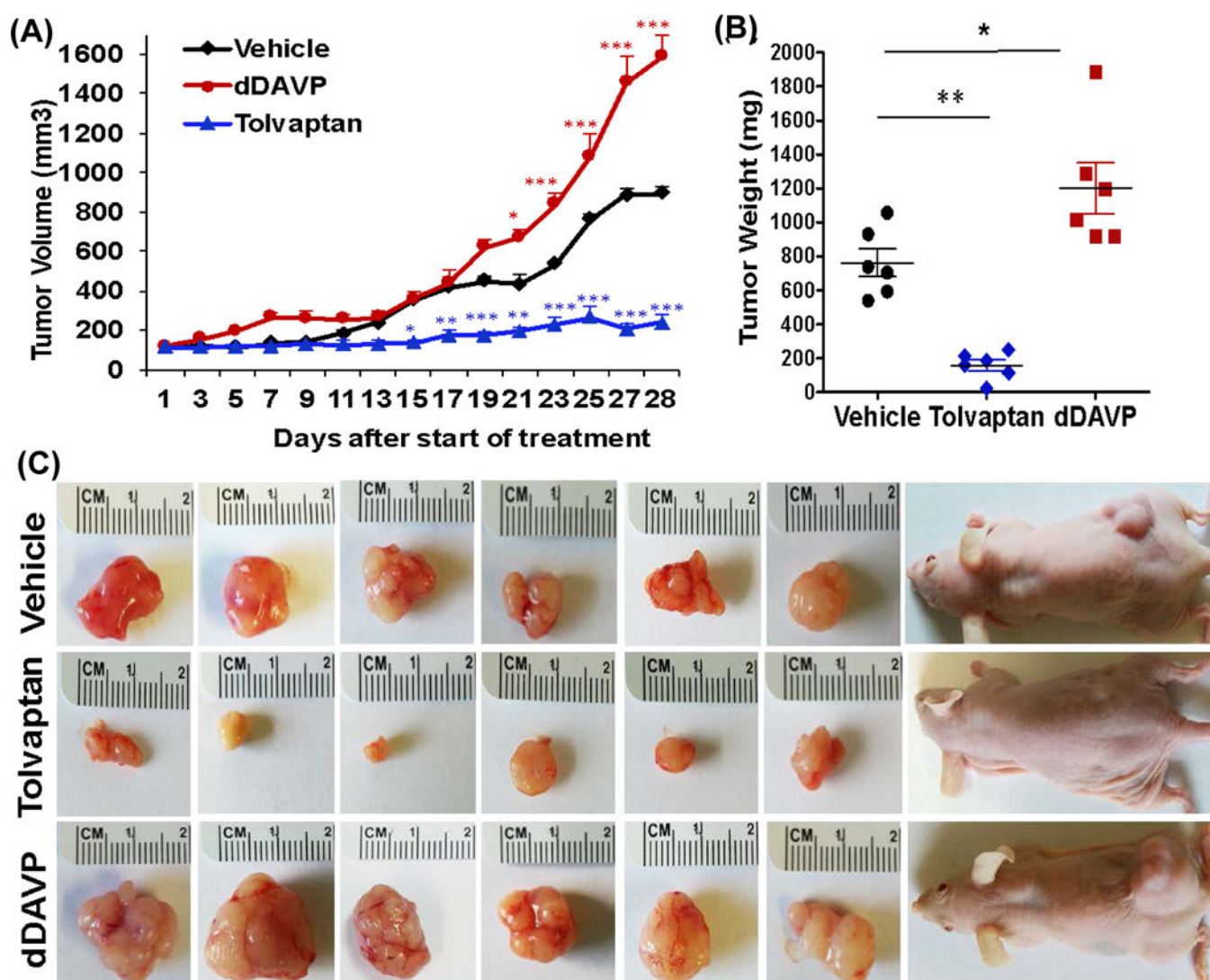


Figure 4. Effect of Tolvaptan or dDAVP treatment on RCC tumor progression:

(A) Nu/Nu mice were inoculated subcutaneously with Caki-1 cells, and 10 days later were treated with vehicle, Tolvaptan (120mg/Kg) or dDAVP (1 μ g/Kg) daily for 28 days. (A) Tumor volumes, (B) tumor weights, and (C) representative images of tumors and mice with tumors at sacrifice (n=6 mice/group). *P<0.05, **P<0.01 vs vehicle (n=6 mice/group). All error bars represent SEM. P values by two way repeated measures of ANOVA followed by the Bonferroni test for Fig 4A and one way ANOVA followed by Dunnett's multiple comparison test for Fig 4B.

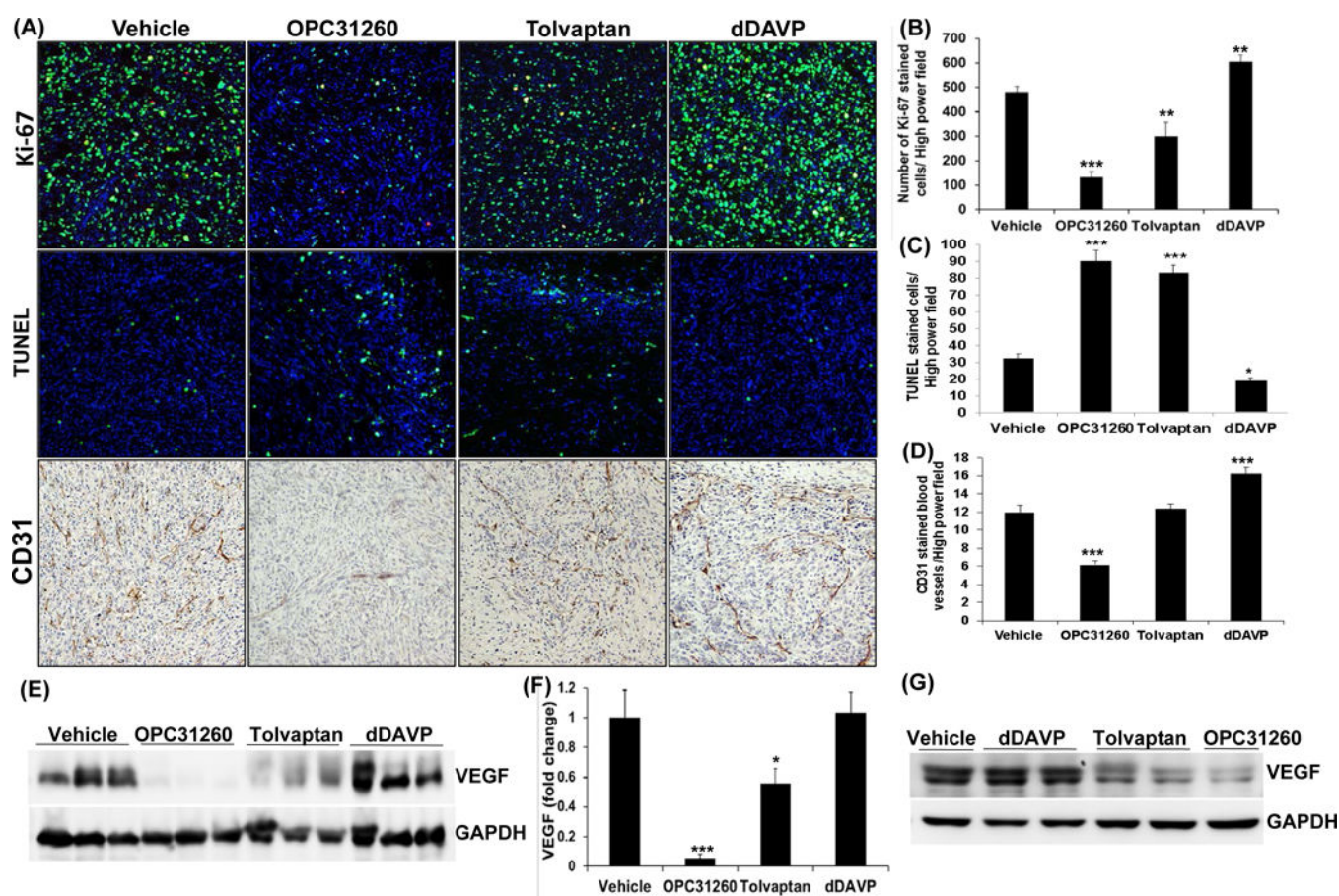


Figure 5. Effect of V2R agonist or antagonists on RCC tumor cell survival and growth: (A) Ki-67, TUNEL and CD31 staining in tumors of mice treated with vehicle, OPC31260, Tolvaptan or dDAVP in tissue sections of tumors from studies in Figures 3 and 4. (B) Quantification of Ki-67, (C) TUNEL stained cells and (D) CD31 stained vessels (n=4 tumors each). (E) Western blot for VEGF and (F) Quantification for VEGF bands by densitometry relative to GAPDH. (G) Western blot for VEGF in Caki-1 cells treated with dDAVP (1nM), Tolvaptan (50μM) and OPC31260 (25μM) *P<0.05, **P<0.01, ***P<0.001 vs Vehicle. All error bars represent SEM. P values by two-tailed unpaired *student's t-test* with Welch's correction and F test in Fig 5B, 5C, 5D, 5F.

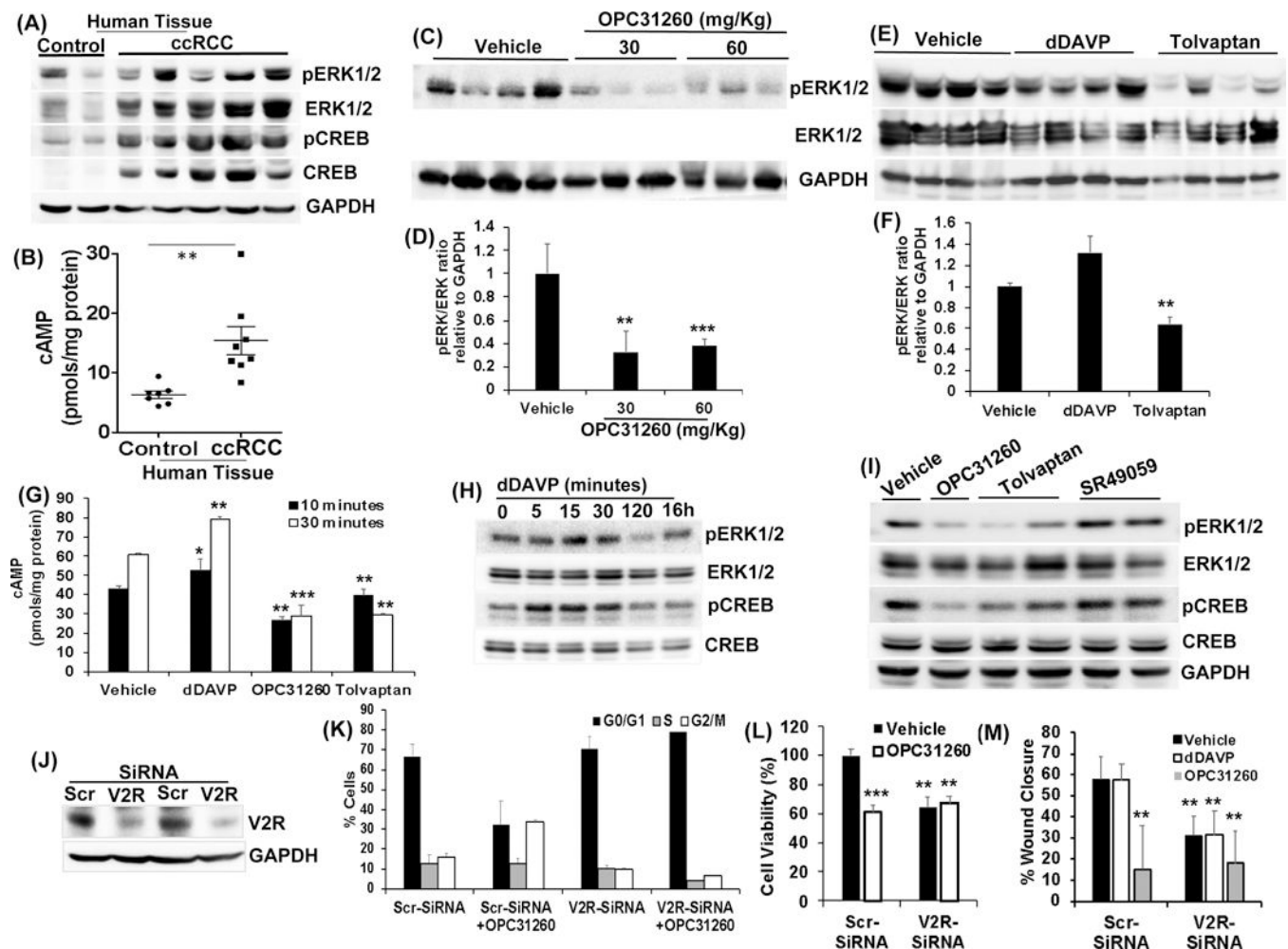


Figure 6. Effect of V2R agonist and antagonists on angiogenesis in RCC tumors:

(A) Western blot using control kidney or ccRCC tumor tissue lysate (B) Intracellular cAMP of ccRCC tumors and control kidney tissue measured by ELISA (n=7 or 8). (C) Western blot and (D) quantification of band density in tumors of Vehicle and OPC31260 treatment groups. (E) Western blot and (F) quantification of band density in tumors of dDAVP and Tolvaptan treatment groups. (G) cAMP levels in Caki-1 cells treated with dDAVP (1nM), OPC31260 (25μM) and Tolvaptan (50μM). (n=4 each). (H) Western blot of Caki-1 cells treated with dDAVP (1nM) or (I) OPC31260 (25μM), Tolvaptan (50μM) and SR49059 (10nM). (J) Western blot for V2R in Caki-1 cells transfected with Scrambled-siRNA (Scr-SiRNA) or V2R-siRNA. (K) In Caki-1 cells transfected with (Scr-SiRNA) or V2R-SiRNA and treated with vehicle or OPC31260 (25μM), cell cycle analysis by FACS and (L) quantitation of % cells in different cell cycle stages. (n=3) (M) Cell viability by MTT assay (n=12) and (N) Percent wound closure by scratch assay (n=12). dDAVP (1nM) was used. *P<0.05, **P<0.01, ***P<0.001 vs control or vehicle in B, D, F and G; vs vehicle treated Scr-siRNA in L, M and N. All error bars represent SD. P values by two-tailed unpaired *student's t-test* with Welch's correction and F test in Fig 6B, 6D and 6F.